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Crassulacean acid metabolism in the seasonally submerged aquatic *Isoetes howellii*

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Summary. Evidence to date is consistent with the hypothesis that the submerged aquatic *Isoetes howellii* Engelmann possesses crassulacean acid metabolism. Quantitative ^{14}C uptake studies indicate that CO_2 assimilation in both the light and dark are functions of pH and total inorganic carbon level. In both the light and dark, maximum uptake rates in 0.6 mM $NaHCO_3$ were double the rates in 0.3 mM $NaHCO_3$. At both carbon levels there was a large drop in carbon assimilation rate between pH 6 and 8. In nature water pH and inorganic carbon level fluctuated diurnally thus complicating the determination of the contribution of light vs dark CO_2 uptake to the total carbon gain. On a sunny day between 0600 and 1200 h water chemistry changed markedly with ~40% reduction in total carbon, ~2 pH unit rise resulting from ~100% depletion of free CO_2. Under such conditions daytime deacidification in *Isoetes* leaves was 88% complete by noon. In contrast, on an overcast day, reduction of carbon in the water was much slower, deacidification was only 46% complete by noon and substantial malic acid levels remained in the leaves at the end of the day. Upon emergence crassulacean acid metabolism was largely lost in *Isoetes* leaves. Preliminary estimates suggest that under natural submerged conditions, early morning photosynthetic rates may be substantially higher than dark CO_2 uptake rates, though uptake rates throughout much of the day could be substantially lower than nighttime CO_2 assimilation.

Introduction

Crassulacean Acid Metabolism has recently been implicated in the submerged aquatic *Isoetes howelliii* (Isoetaceae) (Keeley 1981a, b). The leaves, but not the corms, show a diurnal flux of 100 to 300 μequivalents acidity per gram fresh weight comprised largely of malic acid. $^{14}CO_2$ is fixed in the dark with >85% of the label going into malic acid and gas exchange studies indicate *I. howellii* is capable of substantial net CO_2 uptake in the dark (Keeley and Bowes 1982).

In many CAM plants diurnal acid metabolism is coupled with a diurnal change in stomatal conductance: low conductance during the day and high conductance at night when the water deficit is lower (Kluge and Ting 1978). In these "Super-CAM" plants dark CO_2 fixation contributes the bulk of the total carbon gain. In other CAM plants light and dark CO_2 uptake both contribute to net carbon assimilation and in some CAM plants acid metabolism contributes little to net carbon gain, rather it is involved in recycling respiratory CO_2.

Aquatic plants typically lack stomata (Sculthorpe 1967) although 'amphibious' species like *I. howellii,* that are periodically emergent, generally have stomata. Sculthorpe (1967) contends that stomata on submerged aquatic plants are nonfunctional because of a wax occlusion of the aperature. Light microscopy examination shows the stomata on *I. howellii* are apparently no exception (D.B. Walker personal communication). One piece of evidence that stomata are not involved in acid metabolism is the presence of marked diurnal acidity changes in aquatic *Isoetes* species that lack stomata (Keeley 1982).

Isoetes howellii is capable of net CO_2 uptake in both the dark and light and under similar carbon conditions, light CO_2 uptake is substantially greater than dark CO_2 uptake (Keeley 1981a; Keeley and Bowes 1982). Previously it was suggested that diurnal changes in CO_2 availability under natural conditions may limit daytime carbon assimilation and put a premium on nighttime carbon uptake (Keeley 1981a, b).

The purpose of this study was to examine carbon assimilation rates in *I. howellii* across a range of carbon conditions and to investigate the dynamics of acidification and deacidification in relation to diurnal changes in physical and chemical characteristics of the pools *I. howellii* inhabits.

Materials and methods

Laboratory

Isoetes howellii Engelmann and substrate were collected from the location described below and maintained in aquaria filled with deionized water and kept on a 12 h photoperiod with mid-day photon flux density of 500 μE m^{-2} s^{-1} and 25° C light/15° C dark.

For the quantitative ^{14}C uptake studies, approximately 0.5 g leaf material was harvested and cut into 2 cm segments and split longitudinally, tied into bundles, and weighed. Bundles of leaf material were dropped into 25 ml serum bottles containing 20.0 ml 25 mM buffer (either Na-Citrate pH 5.0, Na-Phosphate pH 6.0, 7.0, or Tris-HCl pH 8.0, 9.0) prepared fresh from boiled distilled H_2O. Mixing was provided by a small stirring bar and excess air space was filled with glass beads. Bottles were stoppered and equilibrated in the light or dark with stirring for 15 min at 25° C. Experi-

ments were initiated by removing 0.5 ml buffer and injecting 0.5 ml $NaH^{14}CO_3$ for final concentration of 0.3 or 0.6 mM $NaHCO_3$ and 0.5 μCi/ml. After 10 min leaf bundles were rapidly rinsed in distilled H_2O and dropped into boiling 80% (v/v) methanol for 5 min. Plant material was homogenized, centrifuged at 10,000 g for 30 min and the pellet washed once with methanol and once with water. The supernatant was evaporated to dryness, resuspended in distilled H_2O, centrifuged and counted. Chlorophyll was determined on a sample of leaf material with the method of Arnon (1949) correcting for small absorption at 710 as suggested by Sestak et al. (1971).

Pool studies

Diurnal acid fluctuations in *I. howellii* leaves were monitored on plants growing in seasonal pools on Mesa de Colorado (610 m), Riverside Co., CA, USA (Kopecko and Lathrop 1975). Soil is a lateritic clay and pools form in the winter wherever depressions are underlaid by a hardpan. During spring 1981, plant and water samples were collected every 6 h for a 48 h period every 2 wk from the beginning of the growing season to pool drying.

Photon flux density was measured with a Li Cor LI-188B integrating meter with a LI-190SB quantum sensor at, and perpendicular to, the water surface and a LI-192SB underwater quantum sensor at the underwater level of the plants. Specific conductance of the water was measured with a YSI-33 conductivity meter at 25° C. Oxygen was determined with a YSI-57 dissolved oxygen meter using a YSI-5700 Clark-type polarographic sensor. A Photovolt 126-A pH meter with combination electrode was used for pH determination and titrations. Carbon dioxide was determined on water samples kept on ice till assayed (usually within an hour) by titrating to pH 8.3 with CO_2-free 0.0227 N NaOH at ambient temperature (APHA 1976). Potentiometric titrations for alkalinity were made on the same samples used for the free-CO_2 determinations using 0.02 N H_2SO_4 titrating to a pH 4.5 endpoint as suggested by APHA (1976).

Plant samples were maintained on ice until extraction, usually within an hour. Leaf samples <0.5 g were washed, blotted dry and weighed on a Ohaus 300 electronic balance run on a car battery. These were ground in a Ten Broeck with 15.0 ml cold CO_2-free deionized water and spun down with a desk top centrifuge run on a gas-powered generator. A 1.0 ml sample of supernatant was deproteinized with an equal volume of 0.6 N perchloric acid and returned to the lab and assayed spectrophotometrically for malic acid with an enzymatic procedure (Gutmann and Wahlefeld 1974). A 10.0 ml sample was immediately titrated with CO_2-free 0.01 N NaOH to pH 6.4 and pH 8.3 which represents the range of values used in studies of acid fluctuations in CAM plants. All data is presented for the pH 6.4 endpoint as this included ~95% of the pH 8.3 diurnal flux (the seasonal range was 94–100%, $\bar{X}=96\%$). Starch levels were determined on plants prepared as follows. Samples were kept on ice 1 to 2 h and then fixed by drying for 1 h at 120° C in a gravity oven. These samples were kept on ice, returned to the lab and dried for 24 h at 60° C. Dried samples were pulverized to pass a 40 mesh screen and assayed colorometrically (Hudson et al. 1976) as modified by Clark and Burk (1980) and further modified by using a sodium acetate buffer of pH 4.5 and an incubation temperature of 55° C.

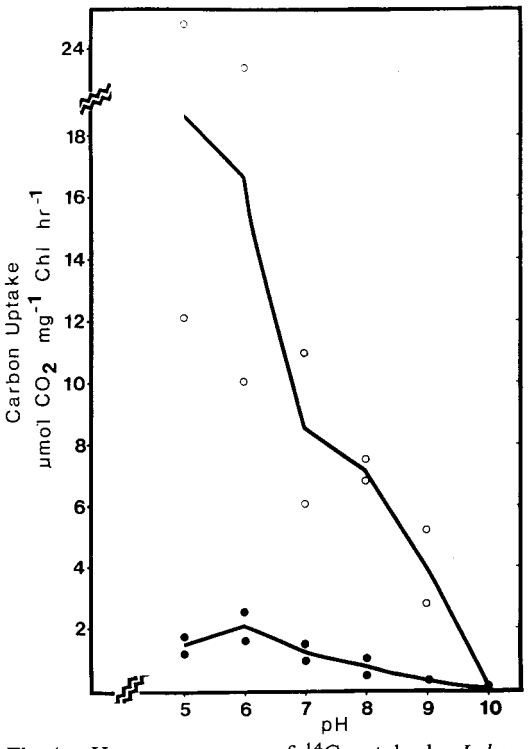

Fig. 1. pH response curve of ^{14}C uptake by *I. howellii* leaves in the light (*open circles*) and dark (*closed circles*) for 10 min in 0.6 mM $NaH^{14}CO_3$

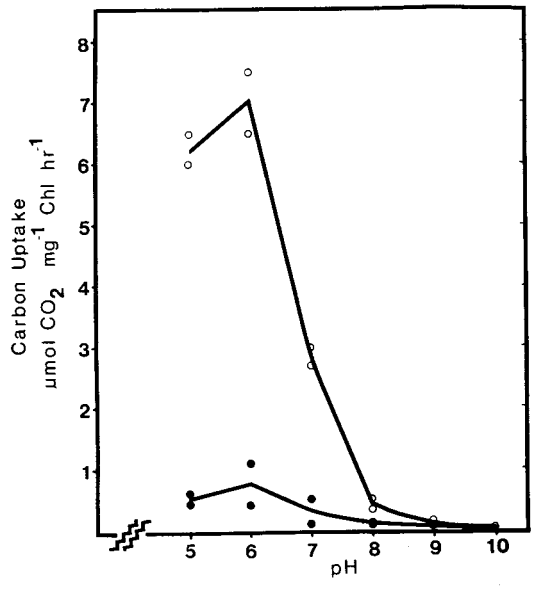

Fig. 2. pH response curve of ^{14}C uptake by leaves in the light (open circles) and dark (*closed circles*) for 10 min in 0.3 mM $NaH^{14}CO_3$

Results

Quantitative studies of ^{14}C incorporation in the light and dark were done in a closed system across the range of inorganic carbon and pH levels encountered under natural conditions. Carbon uptake was a function of both inorganic carbon level and pH. In both the light and dark, maximum uptake rates in 0.6 mM $NaHCO_3$ (Fig. 1) were double the rates in 0.3 mM $NaHCO_3$ (Fig. 2). In the light there was

Table 1. Quantum solar radiation at the water surface, physical and chemical characteristics of the water and titratable acidity (to pH 6.4) and malic acid in *I. howellii* in Mesa de Colorado pool, 4 to 6 April 1981

		Hour						
		1800	2400	0600	1200	1800	2400	0600
Water[b]	QSR (μE m^{-2} s^{-1})[a]	30	0	200	2010	20	0	60
	Temperature (C)	22	15	12	26	23	16	14
	Oxygen (% saturation)	1.6	80	54	151	140	85	49
	pH	7.9	6.7	6.6	8.4	8.6	6.6	6.6
	Free-CO_2 (mg L^{-1})	0.6	3.8	11.5	0.0	0.0	10.5	14.0
	HCO_3^- and CO_2^{-2} (mg L^{-1})[c]	24.6	19.6	25.2	24.1	23.2	24.1	28.8
Submerged leaves	Acidity (μeq g^{-1} FW)[d]	28±1	126±1	211±37	45±4	22±1	104±1	162±20
	Malic acid (μmol g^{-1} FW)	30±3	74±7	117±20	38±1	26±2	65±0	95±12

[a] Underwater quantum solar radiation is shown in Fig. 3 [b] Specific conductance was 80 μmhos cm^{-1}
[c] As $CaCO_3$ [d] $\bar{X}\pm$S.D., $N=2$, FW = fresh weight

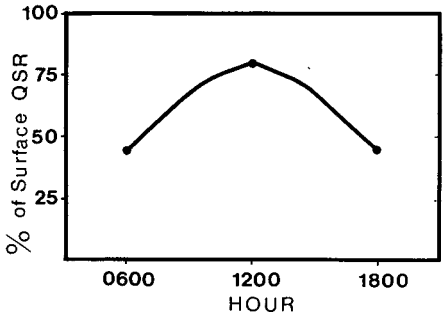

Fig. 3. Underwater quantum solar radiation measured at plant depth (~8 cm) as a percent of surface radiation on 5 April 1981 at Mesa de Colorado

a several fold drop in uptake rate between pH 6 and 8 at the higher carbon level (Fig. 1) whereas at the lower carbon level (Fig. 2) there was an order of magnitude drop in uptake rate between pH 6 and 8. Regardless of the carbon concentration, carbon uptake was ~0 at pH 10. Dark CO_2 uptake showed a clear peak at pH 6 and declined at higher pH.

Diurnal changes in pool chemistry and acid metabolism were studied under natural conditions during spring 1981. Although the pools were filled by late February no *Isoetes* were present during the month of March. The first plants emerged in early April and diurnal acid fluctuations in these plants are shown in Table 1. At noon the quantum solar flux was close to full sunlight at the water surface (Table 1) and the solar flux was ~75% of this at the underwater level of the plants (Fig. 3). Photosynthetic activity by the algal and macrophyte flora contributed to supersaturated oxygen levels in the pools, a depletion of free CO_2 and the concomittant increase in pH. Under these conditions the diurnal acidity change in *I. howellii* leaves was ~200 μequivalents per g fresh weight and the daytime deacidification was 88% complete by noon. Overnight, free CO_2 levels in the water increased and the pH dropped. Nighttime acidification in the leaves was >50% complete by midnight. Throughout the season these patterns were typical for sunny days, though later in the season the water pH did exceed pH 10 in the late afternoon.

In contrast, the patterns observed on an overcast day were quite different (Table 2). On this date photon flux density remained well below half full sunlight throughout the day. This, coupled with the lower temperature, contributed to reduced photosynthetic activity of the pool flora. Oxygen levels only slightly exceeded saturation and substantial free CO_2 levels remained throughout the day contributing to a lower pH. Under these conditions daytime

Table 2. Quantum solar radiation at the water surface, physical and chemical characteristics of the water and titratable acidity (to pH 6.4) and malic acid in *I. howellii* in Mesa de Colorado pool, 17 to 19 April 1981

		Hours						
		1800	2400	0600	1200	1800	2400	0600
Water[a]	QSR (μE m^{-2} s^{-1})	20	0	70	800	300	0	70
	Temperature (C)	21	16	18	16	17	14	13
	Oxygen (% saturation)	113	32	34	103	105	57	40
	pH	7.3	6.6	6.6	7.2	7.2	6.8	6.8
	Free-CO_2 (mg L^{-1})	2.2	9.9	9.5	3.4	3.5	7.9	8.7
	HCO_3^- and CO_3^{-2} (mg L^{-1})[b]	22.0	25.9	29.0	25.5	26.5	29.5	29.4
Submerged leaves	Acidity (μeq g^{-1} FW)[c]	26±3	188±15	271±18	192±8	100±1	172±18	208±11
	Malic acid (μmol g^{-1} FW)	34±2	82±7	143±10	110±5	60±1	86±13	120±1
Emergent leaves	Acidity (μeq g^{-1} FW)	11±1	19±4	28±4	20±4	19±0	27±1	57±12
	Malic acid (μmol g^{-1} FW)	45±6	39±8	45±1	51±8	39±4	46±10	51±8

[a] Specific conductance was 88 μmhos cm^{-1} [b] As $CaCO_3$ [c] $\bar{X}\pm$S.D., N = 2, FW = fresh weight

Table 3. Titratable acidity (to pH 6.4) and malic acid in *I. howellii* from moist and dry soil on Mesa de Colorado, 13 to 15 May 1981

		Hour						
		1800	2400	0600	1200	1800	2400	0600
	QSR ($\mu E\ m^{-2}\ s^{-1}$)	435	–	5	530	77	–	10
	Air temperature (C)	18	–	12	20	14	–	10
Emergent leaves (moist soil)	Acidity ($\mu eq\ g^{-1}$ FW)[a]	6±2	–	12±1	–	4±1	–	10±3
	Malic acid ($\mu mol\ g^{-1}$ FW)	43±4	–	44±2	–	31±2	–	47±6
Emergent leaves (dry soil)	Acidity ($\mu eq\ g^{-1}$ FW)	5±2	–	10±5	–	8±2	–	10±4
	Malic acid ($\mu mol\ g^{-1}$ FW)	44±4	–	50±8	–	29±1	–	55±5

[a] $\bar{X}\pm$S.D., $N=2$, FW = fresh weight

Table 4. Evening and morning levels of titratable acidity (to pH 6.4) and malic acid in leaves of submerged and emergent *I. howellii* in the field and in lab

Conditions			N	Titratable acidity[a] ($\mu eq\ g^{-1}$ FW)		Malic acid[a] ($\mu mol\ g^{-1}$ FW)	
				1800	0600	1800	0600
(Field)	1 May	Submerged	2	13±3	219±8	24±5	124±6
(Field)	14 May	Emergent	4	5±2	11±3	44±4	47±6
(Lab)	19 May	Resubmerged (1 day)	2	2±0	28±6	31±4	43±5
(Lab)	26 May	Resubmerged (1 week)	2	24±8	52±8	7±2	24±5

[a] $\bar{X}\pm$S.D., FW = fresh weight

Table 5. Evening and morning starch levels in *I. howellii* leaves and corms at bi-weekly intervals through the season

		N	Starch[a] ($\mu mol\ g^{-1}$ ODW)			
			Leaves		Corms	
			1800	0600	1800	0600
4–6 April	Submerged	2	144±44	89±17	994±427	688±416
17–19 April	Submerged	2	333±122	72±44	644±550	527±28
	Emergent	2	216±238	0±0	916±150	644±22
1–3 May	Submerged	4	883±211	111±61	560±538	627±211
	Emergent	4	777±677	100±22	2,220±677	3,214±927
13–15 May	Emergent (moist)	4	971±949	44±66	3,897±1,893	2,720±788
	Emergent (dry)	4	627±421	17±22	5,623±2,953	2,254±1,349

[a] As glucose equivalents, $\bar{X}\pm$S.D., ODW = oven dry weight

deacidification in submerged leaves was only 46% complete by noon and substantial acid levels remained in the leaves at the end of the day. These patterns were repeated one other overcast day during the season. On 1 May at noontime, quantum solar flux was 585 $\mu E\ m^{-2}\ s^{-1}$, pool pH = 7.4, free $CO_2 = 3.2\ mg\ L^{-1}$ and daytime deacidification was only 45% complete.

In mid April (Table 2) some plants at the periphery of the pool had their leaves exposed to the atmosphere. These emergent plants had only a very slight diurnal change in acidity. For an equivalent level of acidity emergent leaves had much greater malic acid levels than submerged leaves.

At the end of the growing season, after the pools have dried, plants are exposed to severe soil water deficits. Plants under these conditions showed no tendency for return to diurnal acid metabolism (Table 3). Once again, for an equivalent amount of acidity these emergent leaves had greater malic acid levels than observed for submerged leaves earlier in the season. In fact, throughout the season this difference was quite significant; μeq malic acid – μeq titratable acidity was 26 (S.D. = 12, N, = 21) for submerged leaves and 72 (S.D. = 15, $N = 22$) for emergent leaves, suggesting more of the malic acid pool was in the anion form in emergent leaves.

The plants sampled in mid-May (Table 3) represent conditions at the end of the growing season as all leaves were dead by the end of May. To ascertain the extent to which emergent plants could resume acid metabolism if resubmerged, plants (along with substrate) were collected in mid-May and returned to the lab. Diurnal changes of acidity

in artificially resubmerged leaves did increase over levels observed in emergent leaves but never attained the levels typical of submerged leaves (Table 4). These leaves died within 2 wk of resubmergence and no new leaves were initiated.

Chlorophyll levels in submerged leaves ranged from 0.48 to 0.53 mg g^{-1} fresh weight through the season. In emergent leaves chlorophyll levels increased from 0.61 mg g^{-1} FW in mid April to 0.87 in early May but declined to 0.54 in mid May.

Diurnal and seasonal changes in starch levels in leaves and corms are shown in Table 5. Corms showed no diurnal change in starch levels but seasonal increases were evident. Starch levels dropped markedly overnight in leaves and these diurnal fluctuations were comparable for submerged and emergent leaves. Using a fresh/dry weight ratio of 10.5 (± 2.5, $N=10$) these data were converted to a fresh weight basis for comparison with the overnight changes in malic acid. For submerged leaves the overnight drop in glucose equivalents of starch was 6, 28, and 73 µmol g^{-1} fresh weight for 4–6 April, 17-19 April, and 1–3 May respectively. On these same sampling dates the average overnight malic acid accumulations were 78, 85, and 82 µmol g^{-1} FW respectively. It is clear that early in the season, overnight starch losses cannot account for malic acid accumulation whereas late in the season they are approximately double the level required to maintain acid accumulation.

Discussion

Through the season, overnight malic acid accumulation in submerged leaves averaged ~13 µmol mg^{-1} Chl h^{-1} which is far greater than the maximum rate of dark CO_2 uptake observed in the ^{14}C uptake studies. However, using IR gas analysis Keeley and Bowers (1982) observed dark CO_2 uptake rates for *I. howellii* which were more than sufficient to account for these rates of acid production. Different methodologies used in these techniques may account for this discrepancy. In particular the very high gas flow (1 L min^{-1}) devided into fine bubbles required for gas-water equilibrium in the IRGA studies would tend to produce higher absolute rates of uptake as the stirring utilized in the ^{14}C studies did not produce comparable mixing. Also, in the ^{14}C studies (but not the IRGA studies) the leaves were tied in bundles for rapid removal and thus not all surfaces were equally exposed to the medium perhaps resulting in lower absolute CO_2 uptake rates. Also, if lacunae are not completely exposed by splitting, the delay required to reach ^{14}C: ^{12}C equilibrium inside (Søndergaard and Sand-Jensen 1979) would underestimate uptake rates for the 10 min exposure times utilized in this study. At present it is not possible to say what the absolute rate of dark CO_2 uptake is under field conditions and thus we cannot rule out the possibility that some portion of the overnight acid production many arise from refixation of respiratory CO_2.

It is clear that early in the season daytime starch production is insufficient to maintain overnight acid production. Thus, the substrate for acid production is another carbohydrate, or starch is imported at night from the corms. Later in the season diurnal fluctuations in starch are more than sufficient to account for acid fluctuations, suggesting that CO_2 uptake in the light is important. A similar seasonal pattern in diurnal starch fluctuations is also observed in *Isoetes bolanderi* from oligotrophic lakes (Keeley et al. 1983). As the vernal pools dry down, the emergent *I. howellii* leaves no longer accumulate acids however starch levels still drop markedly overnight. The increased carbohydrate levels in the corms of emergent plants suggests that much of this overnight starch loss from the leaves may be due to export to the corms.

The ^{14}C uptake studies show that CO_2 assimilation in both the light and dark are functions of pH and total inorganic carbon level (Figs. 1 and 2). In the light, CO_2 uptake rates are highest at low pH (where most of the carbon is as free CO_2). Above pH 8 <1% of the inorganic carbon is in the form of free CO_2, thus CO_2 uptake above this pH suggests some capacity for HCO_3^- uptake. However, this capacitiy is dependent on the total inorganic carbon available; at 0.6 mM there is a two fold reduction in carbon uptake between pH 6 and 8 whereas at 0.3 mM there is a 15 fold reduction. It is clear that CO_2 uptake in the light is greatly inhibited above pH 8 and this may reflect very limited HCO_3^- assimilation ability, though direct pH effects cannot be ruled out. Allen and Spence (1981) argue that submerged macrophytes are not easily designated as HCO_3^- "users" or "non-users" but rather for a given carbon level there is a gradation in HCO_3^- use. The results with *Isoetes* are consistent with this hypothesis.

Dark CO_2 uptake has a clear peak at pH 6 and is greatly diminished at higher pH which is interesting in light of the fact that HCO_3^- is the active carbon species used by PEP carboxylase (Kluge and Ting 1978). However HCO_3^- assimilation is apparently an energy requiring process (Raven 1970) and under natural conditions free CO_2 levels reach their highest levels in the pools at night (Tables 1 and 2).

Previously (Keeley 1981a, 1981b) it was hypothesized that crassulacean acid metabolism in a submerged aquatic may have been selected to provide an internal CO_2 source during the day when CO_2 was limiting to photosynthesis. The data presented here are consistent with this hypothesis. *Isoetes howellii* occupies pools which exhibit marked diurnal fluctuations in CO_2 availability of a sufficient magnitude to put a premium on nighttime CO_2 assimilation.

On a sunny day between 0600 and 1200 h, water chemistry changed markedly with ~40% reduction in total carbon, ~2 pH unit rise resulting from ~100% depletion in free CO_2. The inorganic carbon levels used in the uptake studies (Figs. 1 and 2) approximate the range in carbon levels observed in the pools throughout a diurnal cycle. These data would predict the following contribution of light vs dark uptake for a day such as shown in Table 1. In the early morning, carbon levels in the water are high and pH <7; thus light CO_2 uptake rates could be >10 µmol CO_2 mg^{-1} Chl h^{-1} (from Fig. 1). By noon carbon levels are lower and pH >8, thus light CO_2 uptake rates may be <0.5 (from Fig. 2). In fact rates through much of the day could be even lower because the stagnant water conditions typical of these pools produce marked boundary layer effects. This unstirred layer would have a high diffusive resistance to CO_2 since diffusion is several orders of magnitude slower in water than in air. This could have a profound effect on CO_2 uptake rates since it is the conditions near the leaf surface, rather than those in the bulk of the solution, that determine uptake rates. Prins and Helder (1981) showed that the pH near a CO_2 assimilating leaf may be 1 or 2 units higher than in the surrounding medium. They

found that the CO_2 concentration near such a leaf approached the CO_2 compensation point while the CO_2 in solution remained several times higher and this was true for species assimilating bicarbonate as well (Prins et al. 1980).

Overnight, carbon levels in the pools reach their highest level and pH drops <7. This environment would predict dark CO_2 uptake of ~ 2 μmol mg^{-1} Chl h^{-1} or one fifth of the early morning light uptake rates but perhaps many fold greater than the light uptake rates through much of the day.

Other data are consistent with the hypothesis that crassulacean acid metabolism contributes CO_2 under daytime CO_2 limiting conditions. On overcast days when CO_2 depletion in the water is much less, deacidification is much slower and large malic acid pools remain in the leaf at the end of the day. Also, when the pools dry and leaves are exposed to the atmosphere, with its much greater CO_2 diffusivity, crassulacean acid metabolism is largely lost. Consistent with this latter observation is the finding that all 15 aquatic *Isoetes* species so far tested are CAM (Keeley et al. 1981, Keeley 1982, unpublished data) whereas the terrestrial *I. nuttallii* and *I. butleri* have no tendency for overnight acid accumulation even if artificially submerged (Keeley 1983).

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